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General Introduction and Review of Literature

General Introduction

Aquaculture deals with broad spectrum of activities, related to husbanding of aquatic organisms in controlled environments with appropriate propagation methods in the rearing medium, which assures a reliable supply of food. Owing to the highly advanced technology of livestock management, habitat conservation, the challenges of providing food for the ever-growing human population, shrinkage of land area for production and huge investments required for meeting even marginal increase in marine food products, man has turned his attention to aquatic animal farming. The role of aquaculture for augmenting protein food production, improving rural economy and providing large-scale employment opportunities has been well recognized. The increase in demand for cultured prawns, shrimps, fishes, mussels and other aquatic animals had led to research in this area all over the world which has resulted in the development of newer methods of culture and culture practices. Developing countries are the foremost contributors, where aquatic resources are utilized for the livelihood of the population, poverty alleviation, income generation, employment and trade. Aquaculture has emerged as one of the most promising industries in the world with substantial growth. About 63.1% of brackish water production in India is contributed by penaeid shrimp (FAO, 2006). India has an exquisite potential of 1.12 million hectare of potential shrimp farming areas, mainly contributed by vast stretches of highly productive brackish water, and tropical climate favoring faster shrimp growth. Aquaculture production statistics of 2009 describes China with 34.78 million tonnes and followed by India 3.79 million tonnes as the major contributors of freshwater carp and brackish water shrimp production (FAO, 2009).

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The increasing demand for cultured shrimp has led to intense farming practices. Disease loss both by attrition of chronic infection or sudden catastrophic epizootics, poor soil and water quality, high stocking density, accumulation of unutilized feed and fluctuating environmental conditions are the problems confronted by today's aquaculture sector. Though an extensive development of the culture systems has emerged in most of the Southeast Asian countries, successful cultivation is increasingly hampered by environmental pollution, mismanagement, nutritional imbalances, toxicants, stress, diseases and genetic agents. Hence, sustainable development is largely at stake, faced with numerous ecological and pathological problems augmented by environmental degradation and emergence of infectious and non-infectious diseases (Bache' re, 2000).

Disease of aquatic organisms is a major concern. Ecosystems do not respond linearly to environmental changes, nor do the microorganisms that live there. Infectious diseases have distinctive biographies, and each one has a complex relationship with the environment. Complexity of these factors emerges at each level ranging from the cell, organism, community and ecosystem to induce a pathogenic response. Infectious diseases in penaeid shrimp include viral, bacterial, fungal, rickettsial, protistan and metazoan etiologies (Lightner, 1996). The incidence of microbial diseases has increased dramatically in accordance with the growth of aquatic larvae production (Toranzo *et al.*, 1993).

Shrimps are subjected to various diseases and are stressed and weakened under adverse environmental conditions. It was reported that high mortalities occur during first feeding stage of larvae due to emergence of pathogenic and opportunistic bacteria, through food chain (Campbell and Buswell, 1983; Muroga *et al.*, 1987), especially while feeding with *Artemia* (Chair *et al.*, 1994). High density, high nutrient conditions of aquaculture systems facilitate rapid spread of virulent strains. Artificial conditions in aquaculture environments serve as reservoirs for the growth and spread of

pathogenic vibrios. Of the infectious diseases, bacterial and viral infections, either as single or multiple pathogenic conditions, cause extensive production losses.

Viral pathogens reported in shrimp include Monodon Baculovirus (MBV), Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) also called as White Spot Disease Virus (WSDV), Hepatopancreatic Parvo-like Virus (HPV), Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) and Yellow Head Virus (YHV) (Flegel, 1997). Mortalities caused by virus in acute phase can be as high as 95% but surviving shrimp remain infected and become potential source of virus transmission (Kiatpathomchai *et al.*, 2008, Walker and Winton, 2010).

Of the reported bacterial pathogens till date, vibrios are the most important among cultured shrimps responsible for a number of diseases and mortalities upto 100% (Lightner, 1983). Mass mortality caused by luminescent vibrios contributed largely to the collapse of shrimp grow-out activities. Vibrios were isolated from mussels, scallops, oysters, sea urchins, *Artemia*, rotifers, seaweeds, algae, aquaculture market products, from tank-water, seawater, sediments, diseased or dead larvae, and adult organisms (Johan *et al.*, 2003). Internal signs of disease in fish and shellfishes caused by vibrios include intestinal necrosis, anaemia and liquid accumulation in the air bladder, hemorrhages in muscle wall, in or on the internal organs, mouth and or bloody exudates in the peritoneum, swollen intestine, pale mottled liver and gill damage. External symptoms include sluggish behaviour, spiral or erratic movement, damages in the gill and eyes, white and/or dark nodules on the gills and/or skin, fin rot and hemorrhaging at the base. Of the bacterial infection, pathogenicity caused to penaeid shrimps is mainly by vibrios, especially by *V.harveyi*, the diseases commonly referred to as luminous vibriosis. External signs of *V.harveyi* infection in diseased prawns include brittle shells, brown or black spots on the shells, darkened or red body surfaces, pink or brown gills, murky whitish muscle, lack of food

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in the midgut and folded base of the tail (Lavilla-Pitogo *et al.*, 1990, Adams, 1991). Affected larvae develop luminescence, reduced feeding, show sluggish swimming, reduced escape mechanisms, degeneration of hepatopancreatic tissues, formation of necrotic bundles and increased mortality (Robertson *et al.*, 1998).

Several approaches have been proposed to increase aquaculture production, by improving nutritional quality of feed, repress the growth of pathogens in rearing environment (Nogami and Maeda, 1992), treatment with UV, use of nonspecific immunostimulants or vaccines (Anderson, 1992), phage therapy and probiotic bacteria to exclude or inhibit pathogens (Gatesoupe, 1999). The frequent use of high concentrations of these antibiotics poses significant disadvantages like the development of resistant strains and accumulation of antibiotics in crops, thereby causing problems regarding food safety. Antibiotics pose serious threats to human health, by transmitting the resistant microbes from animals to man via the food chain. The most promising prophylactic measure is the use of beneficial or probiotic bacteria (Dalmin *et al.*, 2001). However, several screening methods and field trials are required to select the most appropriate probiotic. Other alternatives are the use of immunostimulants and vaccines which activate the immune system of animals imparting resistance to infections caused by viruses, bacteria, fungi and parasites. Bacterial capsule and extracellular products serve as essential protective antigens, and are effective candidates for vaccine preparations. Bacteriophages are thought to play a major role in the regulation of bacterial population in aquatic environments. Phages are the natural enemies of bacteria, and can be used for biocontrol without interfering with the natural microflora or the cultures in fermented products. The efficacy of phage preparation as therapeutics is reduced by the ever increasing use of antibiotics. The need of the hour is to develop new approaches to control the disease causing pathogens, which are

cost-effective, ecologically sustainable, industrially durable and safe to administer.

Major problem is the diagnosis of pathogenic vibrios from the non-pathogenic benevolent counterparts and eliminating them from the aquaculture systems. However, the hurdles in identification of these environmental isolates are the elevated levels of phenotypic heterogeneity amidst vibrios, inappropriate routine methods of analysis and biochemical variability amongst the species (Vandenberghe *et al.*, 2003). The phenotypic heterogeneity is further mystified by the evidence that some vibrios harbor mobile genetic elements, plasmids and bacteriophages that influence phenotypic characteristics (Munro *et al.*, 2003) leading to continuous revision of the taxonomy of *Vibrio*. Lateral gene transfer (LGT) can result in anomalous placement of a particular taxon, as a result of homologous recombination occurring at intraspecies (*recA*, *dnaE* in *V.cholerae*), interspecies (*asd* from *V.mimicus* to *V.cholerae* non-O₁) and intergenera (*gmd* from *E.coli* to *V.cholerae* O139) levels. New species are being added based on the information gained using various molecular tools which establishes highly informative measure of intra and interspecific genomic relatedness between strains; enabling reproducible and stable classification (Sawabe *et al.*, 2007). Nevertheless, numerical taxonomy of a family, genus or species has its own importance in phenotypically grouping the isolates.

Various virulent factors expressed by vibrios such as adhesion, colonizing factors, extracellular proteases and protective antigens promote their pathogenicity (Austin and Zhang, 2006). A comprehensive search for virulence factors among vibrios revealed unequivocally the role of proteases, lipases, chitinase and plasmid coding for iron chelators apart from haemolysins in initiating an infectious death (Reid *et al.*, 1980, Nottage and Birkbeck, 1987). Non pathogenic and benevolent forms have also been identified to co-exist as part of the natural flora amidst the large number of pathogenic forms. These non- pathogenic forms are essential for nutrient

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cycling, degradation of complex molecules such as chitin. Use of antibiotics, chemotherapeutics etc, have broad spectrum of activity and they are not capable of targeting the pathogenic forms specially and killing them. The treatment with the above mentioned agents kills even the beneficial non-pathogenic forms. Therefore a foolproof diagnostic system to differentiate pathogens from non-pathogenic ones is essential.

Review of Literature

1.1. Distribution of vibrios

Vibrios are widely distributed in aquatic environments from brackish to deep sea waters, commonly found associated with marine organisms and as the important pathogens to farmed animals and human consuming contaminated seafood grown in polluted waters. Vibrios are thought to evolve from marine environments as they require sodium as an important growth factor. Vibrios are frequently detected in summer than winter, probably because they enter into viable but non culturable (VBNC) stage (Barer *et al.*, 1993). Vibrios are frequently found in the digestive tract and on the skin of marine animals. The composition of bacterial population in digestive tract of marine animals differs from that of the surrounding environments, as magnitude of nutrients available in the animal gut is much higher than the surrounding seawater. Healthy *L.vannamei* harbored 10^4 to 10^5 vibrios/g tissue in the hepatopancreas (Gomez-Gil *et al.*, 1998), showing that vibrios also exhibit symbiotic association with the host species. Vibrios attach preferentially to substrates, whereby they colonize and establish themselves. *V.alginolyticus* carries chitin-binding proteins enabling it to adhere to chitin surfaces of copepods and colonize.

Distribution of vibrios in freshwater environments is sparse, as salinity acts as a limiting factor. Vibrios require sodium ions for Na^+ - proton antiports in the energy-transducing cytoplasmic membrane, to maintain cell membrane and cell wall integrity. Some vibrios such as *V.cholerae* can

survive in low salinity, making use of organic nutrients or divalent cations instead of Na⁺. Isolates belonging to family Vibrionaceae obtained from seasonally cold coastal waters indicated variations in morphotypes compared to the other vibrios. This suggests that a large genetic difference in species composition exist among vibrios isolated from seasonally cold or permanently cold environments and their normal counterparts. Distribution and dynamics of *Vibrio* populations are influenced by the biotic and abiotic environment, ecosystems with optimal temperature, salinity, nutrient flow, abundance of host organisms and limited predation stress (Ben-Haim *et al.*, 2003. Heidelberg *et al.*, 2002b)

1.2. Taxonomy of vibrios

1.2.1 Phenotypic characterization of vibrios

Prokaryotic taxonomy deals with the classification (taxa description), identification (strain allocation) and nomenclature of the isolates (Vandamme *et al.*, 1996). Taxonomy of microbes has a sound framework enabling stable, predictable and informative observations. Vibrios are important inhabitants of the riverine, estuarine and marine environments. Vibrios have received the attention of marine microbiologist when majority of the cultured bacterial populations in near-shore waters and those associated with fish and shellfishes were predominantly *Vibrio* spp. (Liston, 1954). The taxonomic group of Vibrionaceae is extremely diverse and can be traced back to the beginning of prokaryotic taxonomy, as vibrios were the first groups of microbes recognized in nature by Pacini, (1854). Shared characteristics of vibrios include NaCl concentration for growth, chitin digestion, morphological features and fermentative metabolism. Phenotypic heterogeneity amidst *Vibrio* spp. make their identification extremely difficult and time consuming especially when conventional bacteriological tests or kits which rely fully upon the phenotypic characters are employed (Vandenberghe *et al.*, 2003; Alsina and Blanch, 1994a, b).

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Currently the family Vibrionaceae has eight genera: *Vibrio*, *Allomonas*, *Catenococcus*, *Enterovibrio*, *Grimontia*, *Listonella*, *Photobacterium* and *Salinivibrio*.

Advent of various molecular tools has resulted in identification of new species based on the sequence information of the house keeping genes including *16S rRNA*, *recA*, *rpoA*, *gyrB*, *gapA*, *ftsZ*, *mreB*, *pyrH*, *toxR*, *23S rRNA* and 16S–23S intergenic spacer region (IGS) (Sawabe *et al.*, 2007). Genetic markers that are unique to a species such as the virulence-associated genes, conserved gene primers and/or probes can be used to quantify the expressed gene and also to determine the taxonomic position. Numerous methods including ribotyping, RFLP, AFLP, RAPD, AP-PCR, ERIC-PCR, PFGE and MLSA are developed for typing and differentiating strains within the same species. Sequencing of the molecular chronometers such as the *5S* and *16S rRNA* has revolutionized prokaryotic taxonomy (Thompson *et al.*, 2005). Additional phylogenetic markers within the 50-100 genes in the bacterial core genome are analyzed to complement the phylogenetic information obtained using the molecular chronometers (Harris *et al.*, 2003). It has been shown that a polyphasic approach based on phenotypic, chemotaxonomic and genomic data, improves bacterial taxonomy and classification (Vandamme *et al.*, 1996) of the genus *Vibrio*. This will most probably increase the number of species in future, as the genus has many new species that are still undescribed (Pedersen *et al.*, 1998; Urakawa *et al.*, 1999a, b; Thompson *et al.*, 2001). Nevertheless, numerical taxonomy of a family, genus or species has its own importance in phenotypically grouping the isolates. Bacterial taxonomy could be performed by sequencing the whole genome, but it is not feasible yet, however, application of MLSA (Multi Locus Sequence Analysis) is a better step towards positioning of vibrios into the varied taxa (Sawabe *et al.*, 2007).

1.2.2. Genomic Characterization of vibrios

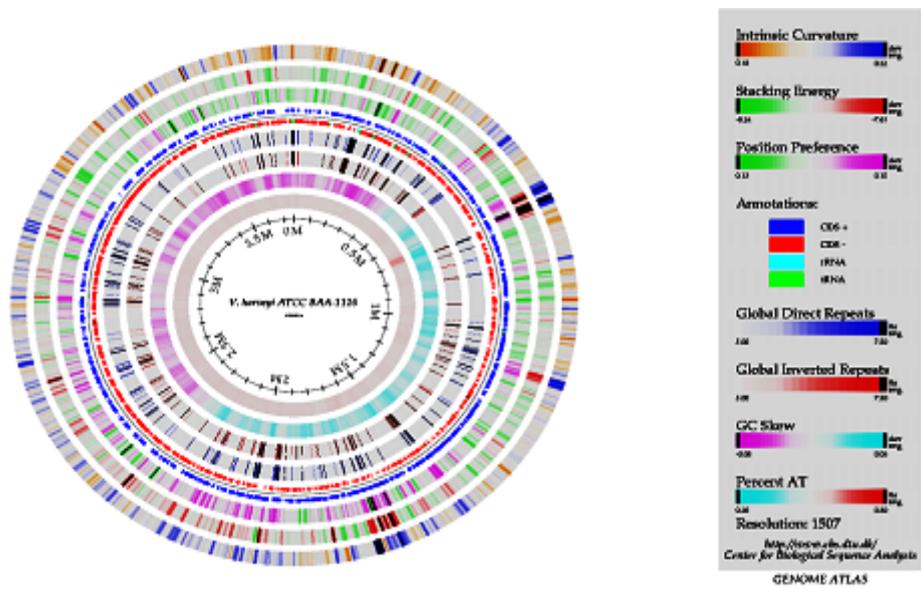
The complete genome sequencing revealed that genus *Vibrio* possesses two circular chromosomes, a large chromosome and a small chromosome. The presence of two chromosomes is common among *Vibrionaceae*, but many do not extend to other families outside this group such as *Aeromonadaceae* and *Enterobacteriaceae*. Okada *et al.*, (2005) suggested that all vibrios have two chromosomes and none of the isolates till date has one chromosome. The presence of essential genes on both the chromosomes, suggests that the small chromosome is an indispensable part of these bacteria (Heidelberg *et al.*, 2000; Makino *et al.*, 2003). The split of the genome into two replicons is advantageous for those bacteria where DNA replication takes place every 8-9min, as in the case of *V.parahaemolyticus* (Joseph *et al.*, 1982). The large chromosome contains genes required for growth, while the small chromosome contains more genes involved in bacterial adaptations to environmental changes, transcriptional regulation and genes coding for transport of various substrates than the large chromosome (Heidelberg *et al.*, 2000; Makino *et al.*, 2003). The small chromosome is thought to have arisen from the large ancestral genome by a single excision (Waldor and Raychaudhuri, 2000). The distribution of functional genes between the large and small chromosomes of vibrios suggests how the two-chromosomal configuration mediates various functions in the organisms and confers evolutionary advantages. The large chromosome contained all the rRNA operons and atleast one copy of all tRNAs, while the small chromosome has intergrons and the third part is the plasmid (Mazel *et al.*, 1998). Examination of the chromosome size in different *Vibrio* species demonstrated that, the size of the large chromosome remained almost stable when compared to the small chromosome, which was variable (Okada *et al.*, 2005).

Both the chromosomes undergo extensive genome rearrangement, however, the location of the conserved regions of either chromosome remains unaltered; suggests interchromosomal rearrangements are less frequent than intrachromosomal rearrangements in *Vibrio* evolution. This view proposed that the ancestral *Vibrio* was diversified into various species retaining the most essential genes in the large chromosome.

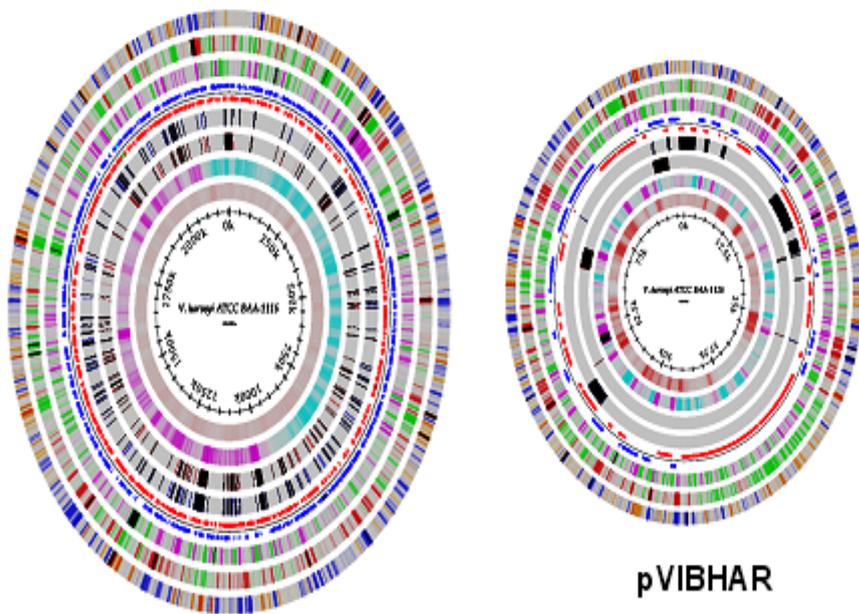
The genome of *Vibrio harveyi* has been sequenced to 8X coverage using a combination of plasmid and fosmid end sequences. The genome has undergone automated sequence improvement (pre-finishing) followed by manual finishing, and automated annotation. The National Science Foundation (NSF) provided funding for the complete sequencing of *Vibrio harveyi* genome. The genome consisted of two chromosomes (Chromosome I and II) and a plasmid (pVIBHAR).

Table 1.1: Details of *V.harveyi* Genome

Characters	Chromosome I	Chromosome II	Plasmid (pVIBHAR)
Accession No:	NC009783	NC009784	NC009777
Basepairs	37,65,351bps	22,04,018 bps	89,008 bps
Genes	3706	2411	120
Proteins Coding	3546	2373	120
Structural RNA	143	24	0
tRNA count	105	16	0
5S rRNA	10	1	0
16S rRNA	10	1	0
23 S rRNA	9	1	0
Pseudogenes	17	14	0
Others	23	11	0
GC content	45.5	45.3	43.8
% Coding	85%	86%	79%



V.harveyi chromosome I



V.harveyi chromosome II

pVIBHAR

Fig 1.2: Genome map of *V.harveyi*
(genome.wustl.edu/genomes/detail/VibrioHarveyi)

1.2.3. Serological characterization of vibrios

Immunological methods for detection of pathogen have been one of the powerful tools used in human and veterinary medicines and application of this technique has percolated into aquaculture also. Serological techniques are used for preparing standard antigens, to obtain antiserum for specific use, purification and labeling of antibodies, which are used in the diagnosis of several diseases and determining the serological properties of major pathogens. Scope and application of these techniques are very high but has to be developed carefully to meet the requirements of aquatic systems. The type and specificity of antibodies produced are direct reflections of the antigens used to produce them. The internal soluble antigen of isolates from the same species tend to be similar but the outer membrane proteins, lipopolysaccharides and capsular antigens tend to be variable (Caugant *et al.*, 1988), that some induce the formation of neutralizing antibody while others induce only binding antibodies.

Serological methods using antibodies targeting the flagellar H (Chen *et al.*, 1992) and LPS (Grisez and Ollevier, 1995) antigens have been developed for the rapid identification of certain pathogenic vibrios. The outer membrane protein-OmpK has been considered as a vaccine candidate for the prevention of infections due to *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in fish. Polyclonal antibody raised against the recombinant OmpK from *V. harveyi* could recognize the OmpK homologues from other strains of *Vibrio* species by immunoblotting. Orange-spotted groupers vaccinated with recombinant OmpK were more tolerant to infection by virulent *Vibrio* strains and their relative percentage survival (RPS) was correlative with the degree of the identity of deduced amino acid sequences of their OmpK (Zhang *et al.*, 2007). OmpK is a conserved protective antigen among tested *Vibrio* species and might be a potential vaccine candidate for the prevention of infections caused by *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*.

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Polyclonal antibody based immunodiagnostic kits for detection of different bacteria (*Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Vibrio alginolyticus* and *Edwardsiella tarda*) in finfish and shellfishes have been developed. However, polyclonal antisera have limitations in terms of cross-reaction, lack of specificity and inability to discriminate antigen at epitope level, and hence monoclonal antibodies (MAbs) are preferred.

Monoclonal antibodies are sensitive to detect antigens at picogram level and scope for false positive is very less as the antibodies detect the existing copies of antigens. Monoclonal antibodies are used for development of simple, rapid and cheap field level tests such as immunoblot for use by the farmers with little training and with simple gadgets. The test is sensitive mostly at 500 picogram level, requiring a detection time of 3hrs for completion, however, it can vary based on the samples. Monoclonal antibodies (MAbs) that recognized distinct species-specific antigenic epitopes which included O-antigens from *Vibrio anguillarum* O2, O2a and certain O2b strains (MAb 7B4) and from *Vibrio ordalii* strains (MAbs A16 and 7D11) were generated. The generated MAbs that react with O-antigens from *V. anguillarum* serotype O1 (MAbs 7B8, 7B5 and 1C3) and serotype O3 (MAbs 13A1 and 14C5) strains (Mutharia and Amo, 2002). These MAbs provide rapid and accurate diagnostic reagents for serological differentiation of *V. ordalii* from serotype O2 strains of *V. anguillarum* (Mutharia and Amo 2002). Monoclonal antibodies (MAbs) developed against four different *Vibrio* spp. that infect humans, fish and shellfish (Phianphak *et al.*, 2005), were tested for their potential application in immunohistochemistry (IHC). Six MAbs (VH1, VH2, VH3, VH4, VH5 and VH6) produced against *V. harveyi* ATCC 14126 were selected. MAb H5 raised against *V. harveyi* ATCC 14126 reacted with all four *Vibrio* spp. as well as against all the *V. harveyi* strains and these were also recognized by MAb H4 and H6. However, MAb H5 recognized 13.5 – 14 kDa bands on Western blot that were not present in the SDS PAGE for the different

Vibrio. More strains of *V. harveyi* from different origins together with non – *Vibrio* species needed to be tested, but it was realized that additional MAbs against *V. harveyi* were necessary to specifically detect all isolates of *V. harveyi* (Phianphak *et al.*, 2005).

Plate and dipstick enzyme-linked immunosorbent assays (ELISA) were developed for the rapid detection of *Vibrio harveyi* from penaeid shrimp and water. The ELISA, which incorporated a polyclonal antiserum produced in a female New Zealand white rabbit, detected 10^5 cells of *V. harveyi*/ml. Also, the systems detected *V. harveyi* in water from Chinese shrimp hatcheries. The systems permitted the recognition of a wide range of *V. harveyi* isolates, but not those of other taxa. Western blot analysis of bacterial outer membrane proteins (OMP) indicated that epitope was recognized, with many immunoreactive bands in common between isolates of *V. harveyi* (Robertson *et al.*, 1998).

1.3. Evolution of vibrios

Variety of events including mutations, chromosomal rearrangements, loss of genes by deletion, gene acquisitions through duplication or lateral transfer are the driving forces for evolution and diversification of bacteria (Makino *et al.*, 2003, Hacker *et al.*, 2003). These factors allow the best adaptive response of the cell within its natural environment (Coenye *et al.*, 2005), also help in tracing bacterial genomes and reconstruction of evolutionary relationships. Mobile genetic elements and lateral or horizontal gene transfer are efficient mechanisms to introduce new phenotypes into bacterial genome (Kurland *et al.*, 2003). Gene duplication involves mechanistic antecedent of gene innovation, leading to genetic novelty, facilitating adaptation to changing environments and exploiting new niches (Hooper and Berg, 2003). Gene duplication and consequent functional divergence are considered as important evolutionary steps, leading to adaptive radiation and broadening the phenotypes.

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An important feature of *Vibrio* genome is the presence of superintegrons. Integrons are natural cloning and expression systems that constitute transferable elements responsible for evolution mainly of multidrug resistance (Rowe-Magnus *et al.*, 2002a, b). Integrase (*intI*) mediates the recombination between a proximal primary recombination site (*attI*) and a target recombination sequence, called the *attC* site (59bps), found associated with a single open reading frame, organized as a circular site termed the gene cassette. Insertion of gene cassette at the *attI* site drives the expression of the encoded proteins. A comparison of the superintegrons of *V.cholerae* and *V.parahaemolyticus* revealed that there is substantial difference between the two gene cassettes (Makino *et al.*, 2003), suggesting that the superintegrons are highly diverse between *Vibrio* species. Chromosomal superintegrons of *Vibrio* might be a genetic source leading to the evolution of resistance to clinically relevant antibiotics through integron-mediated recombinant (Rowe-Magnus *et al.*, 2003). Comparative analysis of the integron integrases, shows that they clearly group together and form a specific clade (Rowe-Magnus *et al.*, 2003). Also all integron Integrase contain a stretch of species specific 16 amino acids located between the conserved patches of tyrosine recombinase family (Messier and Roy, 2001; Nield *et al.*, 2001). Integrons are ancient structure steering evolution by species-specific clustering of the superintegron genes among the bacterial population. This mechanism is seen in vibrios, as systems of gene cluster enabling bacterial adaptation and is termed as *Vibrio* radiation. Comparison of the gene cassette contents between different *Vibrio* species indicates that majority of the cassettes are unique to the host species (Rowe-Magnus *et al.*, 2003). Extensive polymorphism is observed among closely related isolates, suggesting plasticity for these structures and their microevolution through massive Integrase-mediated gene acquisition or loss and cassette rearrangement. Comparison of the nucleotide sequence of vibrios shows that the Integrative and conjugative elements (ICEs) contain conserved set of genes that mediate regulation, excision, integration and conjugative transfer

of the respective ICEs (Beaber *et al.*, 2002a). The proficiency of partnership of integrons and mobile DNA elements is confirmed by marked differences in codon usage among cassettes within the same mobile integrons, indicating that the genes are of diverse origin.

Genomic islands are large DNA regions acquired by lateral gene transfer and inserted into the host chromosomes. The exact character can vary from one island to another, but usual features include insertion near to a tRNA gene, presence of insertion or prophage like elements, flanked by direct repeats and the presence of Integrase gene. Most genomic islands identified in vibrios include virulence related gene cluster, termed as *Vibrio* pathogenic island (VPI). VPI contains pathogenic islands (PAIs) which are regions of bacterial genome, between 10-200kb in length, having characteristic feature of transposable elements, insertional sequence, parts of phages but differing in G+C content and codon usage when compared to the remaining genome. Generation of PAIs often starts with the integration of plasmids, phages or conjugative transposons into specific target genes (tRNA genes), preferentially on the chromosomes (Kaper and Hacker, 1999). On integration into the bacterial genome, these inserted elements experience multiple genetic events, such as mutations, deletions and insertions of genes under specific selective pressure, before resulting in the formation of PAIs (Kaper and Hacker, 1999). Sequencing the PAI genome revealed that this region is widespread in the bacterial genome, offering evolutionary advantage. PAIs and superintegrons score over mutations in bacterial evolution, as entire gene clusters or operons are transferred and incorporated in the host genome, resulting in a dramatic change in the host behavior (Groisman and Ochman, 1996).

Plasmids are diverse in vibrios and are used in differentiation of strains within a species, by a technique called Plasmid profiling. *Vibrio* plasmids vary in size ranging between 0.8 to 290kb, even within a single serogroup of a specific species. The frequent identification of prophage

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DNA in *Vibrio* chromosomes shows how widespread are plasmids in microbes, enhancing the chances of mobilizable plasmids along with their conjugative counterparts. On a smaller evolutionary scale, intraspecific and interspecific homologous recombination takes place between vibrios. The number of nucleotide substitutions caused by recombination versus point mutation is in the ratio 3:1, influencing the microevolution of *Vibrio* genome.

VHML (*V.harveyi* Myovirus like) infected *V.harveyi* are not able to hydrolyze L-glutamic acid 5-(4-nitroanilide), indicating a lack of operational glutamyltranspeptidases, whereas the uninfected strains could hydrolyze this compound. The variability in phenotypic profile indicates that VHML integrates into the host genome and causes changes in the phenotypic profile of the organism, causing the misidentification of *V.harveyi* isolates (Vidgen, 2006).

The evolution of virulence in mutualistic associations: Symbiosis among Vibrionaceae occurs with many marine host species, especially in vibrios colonizing crustacean (Bowser *et al.*, 1981), mollusc (McFall-Ngai, 2002), or fish hosts (Schiewe *et al.*, 1981; Wiik *et al.*, 1989; Toranzo and Barja, 1990). Although a number of these pathogenic vibrios have common physiological attributes, it has always been a question of whether virulence or virulence factors (i.e., pathogenicity islands) were common among the symbionts. Investigations assaying biochemical features (Lunder *et al.*, 2000), iron sequestration (Tolmasky *et al.*, 1985), and plasmid profiling (Sorum *et al.*, 1990) grouped many of the pathogens together, according to their specific hosts that they infect. Although this may provide a “common ground” for all species studied, 5S and 16S rRNA molecular data provide evidence that most of these alliances are not robust (Wiik *et al.*, 1995) and the pathogenic species of *Vibrio* are not monophyletic. This is probably due to the fact that most phenotypic characters are more likely to place species

or species groups according to the type of habitat and the abiotic factors that influence the phenotype of that particular species or strain (Cohan, 2002).

1.4. History of *V.harveyi*

V.harveyi was first described as species of *Acromonobacter* by Johnson and Shunk in 1936. Later on this bacterium was grouped along with other luminescent bacteria under the name *Beneckea harveyi*. In 1981, Baumann *et al.*, abolished the names *Beneckea* and *Lucibacterium* and transferred it into *Vibrio* based on its characteristic shape. With the advent of large-scale prawn culture, *V.harveyi* got attention as a shrimp and prawn pathogen, particularly in tropical areas. *V.harveyi* is very closely related phenotypically and genotypically to *V.carchariae* that the latter strain is considered as a junior synonym of *V.harveyi* by Gauger and Gomez-Chiarri (2002). Great diversity of *V.harveyi* poses certain difficulties in the biochemical determination and identification of environmental vibrios. Identification and typing of *Vibrio* strains using genomic approaches and ribotyping are useful for taxonomic studies and identification to the subspecies level (Austin *et al.*, 1995). The two central members of the *Vibrio* core group which are closely related include *Vibrio campbellii* and *Vibrio harveyi* which are known to thrive in similar environments and share a high degree of genetic and phenotypic similarity. *V. harveyi* strain CAIM 1792 provides important insights into the metabolic capability, pathogenicity and genetic plasticity of each and aid in adjusting the attribution of certain characteristics (e.g. bioluminescence, obligate organoheterotrophy) that have previously been used to define *V. harveyi* and *V. campbellii*.

Outbreaks of vibriosis have been reported worldwide, however, *V.harveyi* causes disease in a variety of aquatic organisms, including marine fish, bivalves and crustaceans. Infections in fish are mostly as opportunistic pathogen or through stress in captive environment than report of disease in

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invertebrates. Most *V.harveyi* strains are not harmful to larvae of *P.monodon*; however, some strains are extremely pathogenic. Symptoms exhibited by *V.harveyi* on fishes include anorexia and darkening of the whole fish, along with appearance of local hemorrhagic ulcers on mouth or skin surface and focal necrotic lesion in the muscle or eye opacity. Lavilla-Pitogo *et al.* (1998) reported epizootic of luminescent, non-sucrose-fermenting *V.harveyi* in larvae of *P.monodon* in Philippines. Luminous vibriosis is the widely used term for mortality caused by *V.harveyi* in penaeid prawns. *V.harveyi* enters the larval prawn through mouth and feeding apparatus, and usually found colonizing the oral cavity of the larvae (Lavilla-Pitogo *et al.*, 1990). Infections caused by *V.harveyi* are usually septicemic, with the pathogenic agent being isolated from the hemolymph and hepatopancreas of infected animals (Liu *et al.*, 1996 a, b). Increase in amounts of organic matter in ponds, tanks and use of contaminated equipment between ponds are the probable factors for *V.harveyi* spread. Aerosol transmitted contamination by *V.harveyi* of Marine algal cultures given as feed, Artemia cysts carrying *V.harveyi* or from cross contamination from workers hands or equipment are considered as other causes of vibriosis (Owens, 2006). The ability of *V.harveyi* to utilize a wide variety of organic compounds as carbon and energy source aids the survival of this specie when competing for scarce nutrients present in the marine environments (Ramesh *et al.*, 1989). Variation in environmental conditions of the susceptible host, particularly when raised under intensive cultures with cold temperatures, overcrowding and inadequate water circulation, facilitate the outbreak of the disease, thereby causing massive destruction to aquaculture industries.

1.5 Bacterial Adaptations

1.5.1. Biofilm formation

Majority of bacteria have the biofilm forming property, which involves the assemblages of bacteria on a surface encased by an

extracellular matrix, rather than as free-swimming entities (Costerton *et al.*, 1978). Bacteria within the biofilm show increase in resistance and metabolic efficiency of the population, compared to their planktonic counterparts to variety of stresses, including UV, acidic conditions, dehydration, oxidative environment and antimicrobial agents (Jefferson, 2004). Biofilm-mediated attachments to abiotic and biotic surfaces are important for survival of *Vibrio* spp. Most vibrios show attachment to copepods, crustaceans, insects, plants and filamentous green algae using the property of biofilm formation (Hood and Winter, 1997; Bourne *et al.*, 2006). The ability to attach to external and mucosal surfaces is an important virulence determinant of bacteria. Protozoan grazing is identified as one of the key biotic pressures faced by bacteria, which is overcome by the formation of microcolonies or flocs. *Vibrio* species may use marine animals as vehicles for survival when encountered with protozoan grazing pressure.

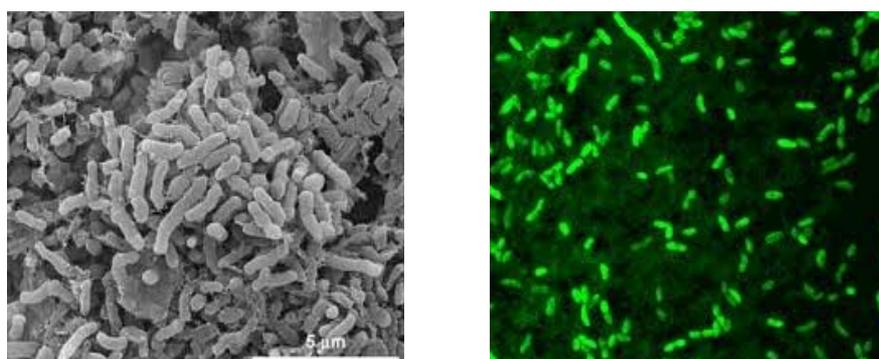


Fig 1.3: Bacterial assemblage for Biofilm formation

(www.scoopweb.com)

In response to this pressure, bacterial communities develop inedible phenotypes, referred to as grazing-resistant varieties; this adaptation brings about profound changes in the structural and taxonomic position of the communities (Matz *et al.*, 2002b). Protozoan grazing is considered as one of the selective forces in evolution of pathogens, as bacteria develop various virulence factors as adaptive measures to protect themselves against predation.

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1.5.2. Capsule and EPS

The production of capsule and *Vibrio* exopolysaccharides (EPS) are of relevance during infection and resistance to environmental stresses (Costerton *et al.*, 1978, 1981). The opaque or rugose cells are more resistant to infection compared to their translucent or smooth counterparts. The capsule in the rugose cells helps to evade phagocytosis and switch to the smooth stage for dispersal and colonization of new sites. Vibrios have the ability to switch from encapsulated to unencapsulated morphotypes based on the environmental niches they occupy. In addition to the genes necessary for the capsule and EPS production as response to varied environment, these genes are also involved in biofilm formation (Kierek and Watnick, 2003a). Elevated level of intracellular cytidine leads to increase in EPS production and thus biofilm formation. Quorum-sensing (QS) regulates biofilm formation and influences attachment to biotic surfaces in a number of *Vibrio* species (Hammer and Bassler, 2003). QS repression by HapR, flagellum-regulated repression of EPS and increased EPS regulation are seen in rugose morphotypes. The presence of multiple signaling pathways for regulating EPS and biofilm formation indicates that different pathways operate in diverse environments or selection of different strains occurs under certain conditions (Heithoff and Mahan, 2004). Vibrios are found to possess mannose-sensitive hemagglutinin (MSHA) pilus which enables their attachments to cellulose, but was not required for biofilm maturation. Vibrios have similar or overlapping mechanisms regulating attachment to chitin and other surfaces in seawater favoring bacterial colonization. Evolution of new phenotypic traits enhances the attachment and colonizing behavior, surreptitiously increasing the ability of the bacteria to invade host organisms.

1.5.3. Starvation adaptation mechanism

Vibrios exhibit an elaborate and highly developed starvation adaptation mechanism, by altering the gene expression as well as physiological changes for survival in unfavorable conditions (Kolter *et al.*, 1993). Vibrios adapt to starvation stress by reducing its cellular volume, DNA and ribosomal content and the rate of protein synthesis (Ostling *et al.*, 1993). First stage of starvation adaptation is governed by the accumulation of guanosine 3'-diphosphate 5'-diphosphate (ppGpp), followed by the shutdown of macromolecular synthesis, increased rate of protein degradation and reorganization of cellular components (Cashel *et al.*, 1996). Second stage is the decrease in ppGpp and increase in the macromolecular synthesis, followed by shifts in fatty acid composition of the membrane, degradation of reserve materials and activated resistance development towards a variety of stress (Wong and Wang, 2004). Third phase again involves the gradual decline in macromolecular synthesis and metabolic activities, such as endogenous respiration, modification to tolerate and survive in stressed environment until the emergence of favorable conditions. During starvation, specific proteins related to peptide chain elongation, protein folding, carbon metabolism and stress resistance exist in oxidized state, leading to the formation of aberrant proteins owing to microincorporation of aminoacids (Dukan and Nyström, 1999). Reduction of translation accuracy is caused by ribosomes which are starved for the cognate tRNAs, resulting in protein degradation in starved cells (Nyström, 2004). Starvation induced proteins (Sti) are synthesized in the initial starvation phase, as these proteins offer protection against external stress such as heat, osmotic stress and oxidation (Dukan and Nyström, 1999). Thus, making the starved cells resistant to a variety of stresses is termed as starvation induced cross protection (Jenkins *et al.*, 1990).

Vibrios can tolerate carbon shortage for a month or longer, making use of the carbon stored in the inclusion bodies as reserve of glycerol or

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poly 3- hydroxybutyrate. Carbon limitation and hike in cAMP levels stimulate protease activity in vibrios, mediating both detachment from surfaces and penetration into mucus layers during tissue colonization (Benitez-Nealson, 2000). Carbon starvation results in both energy and nutrient limitations, while nitrogen and phosphorus starvation do not cause cessation of growth. Bacteria still continue to grow, utilizing the intracellular reserve of nitrogenous polymers (Mason and Egil, 1993). Similarly, inorganic polyphosphates is essential for adaptation to stress and survival in stationary phase (Rao and Kornberg, 1996). Starvation induction is mediated by several regulators including σ factor, RpoS in many species (Lange and Hengge-Aronis, 1991). Bacteria have evolved complex mechanisms to cope up with the environment induced stress, characterized by changes in gene expression, physiology and morphology.

1.5.4. Viable but nonculturable response (VBNC)

Vibrio spp. during prolonged unfavorable conditions enter a stage where the cells become incapable of undergoing cellular division on the normal growth supporting media but remain metabolically active (Oliver, 1993; Rice et al., 2000). During environmental stress such as starvation, salinity variations, variations in visible light and/or temperature differences, bacteria enter the VBNC state (Lee and Ruby, 1995). VBNC cells have a thickened periplasmic space to resist heat, cold or desiccations. However, bacteria exhibiting loss of cultivability and reproducibility under stress conditions revert to normal state breaking the period of dormancy when the conditions become favorable. Stasis is caused by a variety of conditions that induce the expression of regulators involved in the prevention and repair of damages caused to cellular components. VBNC population exhibits a decrease in superoxide dismutase activity, resulting in an increase in oxidative damage and induction of stress regulons, such as those regulated by RpoS and RpoE.

1.5.5. Other adaptations:

Vibrios are well adapted to live in the gut of marine animals, establishing themselves in the hepatopancreas, hemolymph and digestive tract. Vibrios have developed mechanisms for tolerating low pH, secreted bile acids and anaerobic environments. Once inside the gut, vibrios colonize the gut of the host by overcoming and adapting itself to the host defense mechanisms, especially those preventing bacterial invasion and growth. High substrate affinity of vibrios suggests adaptation to growth under high-nutrient conditions occurring in host gut or in planktonic microenvironments. Respiratory activity under low-nutrient conditions in seawater mesocosms, indicates long term survival of vibrios in substrate limiting environments (Armada *et al.*, 2003). Maintenance of high ribosomal content after shift from starvation stress enables a rapid growth in response to favorable conditions (Pernthaler *et al.*, 2001). Chemotaxis towards chitin, sugar monomers, amino acids and response to limited concentration of carbon, indicates the ability of vibrios to exploit nutrient-rich microenvironments (Bassler *et al.*, 1991; Larsen *et al.*, 2004). ToxR and to a lesser extent ToxS enhance resistance of *Vibrio* to bile, and bile in the growth medium increases expression of OmpU, which helps vibrios to tolerate high bile concentration in the host (Wang *et al.*, 2003).

1.6. Virulent Factors of vibrios

Vibrio spp. show great variation in terms of pathogenicity associated with host species, its developmental stage, bacterial dose, bacterial species and particular strains, and exposure time and stress (Lightner, 1996; Saulnier *et al.*, 2000a; Aguirre-Guzmán *et al.*, 2001).

1.6.1. Extracellular products

Different *Vibrio* extracellular products (ECP) have been identified and proposed as putative virulence factors in the species pathogenic to

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shrimp (Liu *et al.*, 1996, 1997; Lee *et al.*, 1997a, 1999; Chen *et al.*, 1999, Harris & Owens, 1999; Montero & Austin, 1999). A thermo-labile cytotoxic factor was detected in the ECP from *V. penaeicida*, which produces 100% mortality in juvenile *Litopenaeus stylirostris* (Goarant *et al.*, 2000). Proteolytic enzymes, such as cysteine and serine proteases, metalloproteases, and hemolysins, have been isolated from *Vibrio harveyi*, *V. anguillarum*, and *V. alginolyticus* (Lee *et al.*, 1997a; Harris & Owens, 1999). *V. harveyi* produces an extracellular 38 kDa protein with protease, phospholipase, and hemolytic activities for *Penaeus monodon* (Liu *et al.*, 1997). Zinc metalloprotease Emp, secreted as a 48kDa proenzyme is implicated as a virulence factor in *V.anguillarum* (Staroscik *et al.*, 2005). *V.harveyi* produces proteases, phospholipase, hemolysins or exotoxins important for pathogenicity (Liu *et al.*, 1996). Bacterial haemolysin has been suggested as an important virulent factor of pathogenic vibrios (Chang *et al.*, 1996). Therefore, haemolytic assay has been used in the differentiation of virulent strain among suspected pathogens (Chang *et al.*, 1996).

An exoprotease has been purified from the extracellular product of *V.harveyi* 820514 by a combination of ammonium sulphate precipitation, hydrophobic interaction chromatography and anion exchange chromatography on fast protein liquid chromatography. Purified protease appears to be a cysteine protease by virtue of the inhibition of enzyme activity, iodoacetamide, iodoacetic acid, N-ethylmaleinide, p-chloro mercuribenzoate. It is the first cysteine protease found in *Vibrio spp.* Cysteine protease is a major exotoxin lethal to *P.monodon*, interfering with hemostasis, leading to formation of unclottable hemolymph (Liu and Lee, 1999). A thermostable exotoxin of *V.harveyi* having proteolytic, hemolytic and cytolytic activity was recovered from diseased postlarvae of *Penaeus vannamei* (Montero and Austin, 1999). ECPs from *V.harveyi* VIB 645 containing caseinase, gelatinase, phospholipase, lipase and hemolysins with

high titre of hemolytic activity to salmonids erythrocytes were determined by Zhang and Austin (2000).

1.6.2. Adhesins and Outer membrane proteins

Animal-bacterial cell interactions are often maintained by the recognition of the sugars on the host cell membrane by bacterial surface proteins called Adhesin (Costerton *et al.*, 1978, 1981). Many Gram –ve bacteria have mannose – recognizing adhesins and specificity of interaction is conferred by variations in the bacterial adhesins that corresponds to differences in the microenvironment of the mannose residue on the host receptor. The outer membrane proteins are encoded by *OmpU* functions as an adhesin. Outer membrane proteins (Omps) called porins participate in adhesion to host. Adherence of *V. cholerae* to a variety of cell lines *in vitro* and colonization of infant mice are inhibited by Fab fragment from anti-*OmpU* antibodies (Provenzano and Klose, 2000; Simonet *et al.*, 2003).

1.6.3. Lipopolysaccharides

The most common bacterial inducer of animal cell death is bacterial LPS and specifically, the lipid–A portion of LPS, which is the most conserved component of the molecule (Nikaido, 1988; Nesper *et al.*, 2000). LPS of bacteria inhibits further cell proliferation and induces cell death. The opportunistic pathogen, *Vibrio vulnificus* expresses lipopolysaccharide antigens on its outer membrane surface. Five O-antigen- specific MAb were used to detect distribution of the serotypes among *V. vulnificus* strains isolated from various settings. While a number of *V. vulnificus* strains were unrecognized by the five MAb, and some strains were recognized by more than one MAb, the application has proven useful in demonstrating O-antigen distribution in both clinical and environmental isolates (Zuppardo *et al.*; 2001). Montero and Austin (1999) suggested that the LPS might constitute the lethal toxin of *V.harveyi E₂* to penaeid shrimp.

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1.6.4. Flagella as chemotactic and virulence agent

The two flagellar systems operate to propel bacteria under different circumstances. The polar flagella aids in swimming and lateral flagella in swarming are composed of multiple flagellin subunits, sheathed by a membrane and rotate by using energy derived from the sodium membrane potential. The presence of peritrichous flagella functional in viscous environments enables bacterium to move over and colonize surfaces (McCarter, 1999). Flagellar navigation brings about chemotaxis response in vibrios enabling them to move away from unfavourable environments, a response important for bacterial survival and colonization. Motility and chemotaxis have shown to play the role in virulence of *V. anguillarum* (Larsen and Boesen, 2001). Antigenicity of lateral flagella of different species differs from each other, except for the lateral flagella of *V. parahaemolyticus* and *V. alginolyticus* which share the same epitopes. Also two kinds of antigenic determinants or sites are present. One is an antigen on the surface of the intact flagella and the other located inside the flagella which becomes exposed when flagella are solubilized to flagellin monomers. Thus confirmed that *V. parahaemolyticus* is divided into three types HL1, HL2 and HL3 and they showed no cross reactivity with H-antigens of the serotype of *V. parahaemolyticus* and other strains. (Shinoda *et al.*,1976). Flagella consists of flagellinA, essential for virulence and the expression of *virB* and *virC* genes, responsible for production of major surface antigens, located on the outer sheath of flagellum, important for virulence (Norqvist and Wolf-Watz,1993). The chemotaxis genes (*che*) are differentially regulated within *Vibrio* spp. and mutation to this region results in different rotational biases and profound difference in colonization exhibited by the bacterium. Many pathogenic *Vibrio* species are attracted towards mucus, enabling their colonization in the intestinal mucus as seen in *V. alginolyticus* infection to fish (Bordas *et al.*, 1998). The genome sequence of *Vibrio* species reveal a plethora of potential chemoreceptors found distributed on both the

chromosomes. The genes mainly identified include the methyl-accepting chemotaxis protein (MCP) genes, involved in sensing and responding to varied environmental signals (Gestwicki *et al.*, 2000). Flagellar motors participate in signal transduction cascade, influencing the expression of cell surface polysaccharide, which mediates important function such as biofilm formation and host colonization (Watnick *et al.*, 2001; Lauriano *et al.*, 2004).

1.6.5. Type Three Secretion System

The type three secretion systems (TTS) enable many pathogenic Gram negative bacteria to directly inject eukaryotic cells using fibrous structures on bacterial surface called injectisomes. TTS forms an important part of the *Vibrio* pathogenic islands, mediating virulence. Structural components of TTS are highly conserved between different pathogenic species (Park *et al.*, 2004). Bacteria using this mechanism share at least 8 genes and many have over 20 components that are essential for proper functioning. Certain species can be artificially induced for TTS for substrate recognition, by growth at 37°C in the absence of calcium, causing protein secretion into the media. The signals for protein secretion are located on the first 15 codons of the ORF. mRNA signals TTS export by coupling its translation with the secretion of encoded polypeptide. mRNAs are eventually relieved from folded structure for a productive interaction between charged ribosomes and TTS machine and the proteins are secreted across the bacterial envelope in a Co-translational manner. Henke and Bassler (2004) reported a functional TTSS in *V.harveyi* governed by TTSS genes which is homologous to those found in *V.alginolyticus* and *V.tubishii*. Different vibrios use different kinds of natural targets for protein injection by TTSS, enabling a better understanding of the lifecycle of vibrios in natural environments.

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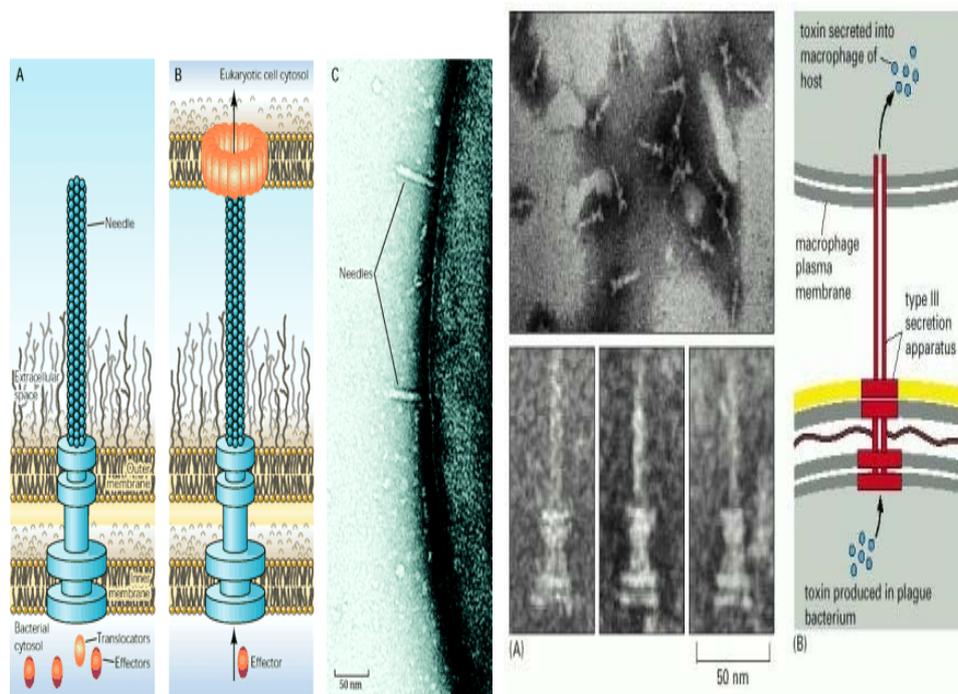


Fig: 1.4 Type three secretion system governed by *V.harveyi* injectisomes
(physiologyonline.physiology.org/content/20/5/326F1.expansion.html)

1.6.6. Integron mediated resistance

Integrans are natural genetic engineering platforms that incorporate ORFs and convert them into functional genes, ensuring correct expression. All integrans are characterized by 3 key elements necessary to produce functionally effective exogenous genes: a) gene coding for an Integrase of tyrosine recombinase family (*intI*), b) a primary recombination site (*attI*), and c) a strong outward-oriented promoter (P_c). Integrans are able to capture one or more gene cassettes from the environment and incorporate them by using site- specific recombination. The integron Integrase only mobilizes the gene cassettes within the integrans. The role of integrans and gene cassettes in dissemination of multidrug resistance in Gram-negative bacteria is well established (Hall and Strokes, 1993). Based on the integrase gene sequences, at least eight different classes of integrans have been described in Gram-negative bacteria (Nield *et al.*, 2001). Class 1 integrans

are found associated with functional transposons such as Tn21 (Liebert *et al.*, 1999) and Class 2 integrons inside Tn7 derivatives (Radstrom *et al.*, 1994). Class 1 integrons found in clinical isolates mainly govern multidrug resistance; contribute to the spread of genetic determinants of antibiotic resistance by horizontal gene transfer, although not mobile elements themselves, they are frequently associated with plasmids and transposons (Fluit and Schmitz, 1999). Integrase gene of Class 1 integrons (IntI) code for site-specific recombinase responsible for cassette insertion (Collis *et al.*, 1993) along with the attI site where the cassettes are integrated and a promoter (Pc) enables the transcription of the cassette-encoded genes, hence these two are suspected as the reservoirs of antimicrobial resistance genes within the microbial populations (Pai *et al.*, 2003). The increasing incidence of integrons and other resistance determinants among veterinary microorganisms reduces therapeutic options for both human and animal diseases due to an increased prevalence of resistant zoonotic pathogens, which could subsequently cause human infections during processing and preparation procedures (Hopkins *et al.*, 2005). Exchange of genes for resistance to antibiotics between bacteria in aquaculture environment and bacteria in terrestrial environment, including bacteria of animal and human pathogens has been shown by Schmidt *et al.*, (2001). Many classes of antimicrobial agents, such as aminoglycosides, chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole have been reported as active antimicrobials (Zhao *et al.*, 2001). Five different classes of mobile integrons are involved in the dissemination of antibiotic resistance genes. All five are physically linked to mobile DNA structures, either associated with insertion sequences, transposons and/or conjugative plasmids, serving as vehicles for intra-and inter- species transmission of genetic material. Class 1 integrons confer resistance to all β -lactams, aminoglycosides, chlroramphenicol, trimethoprim, streptothricin, rifampin, erythromycin and antiseptics of quaternary ammonium compounds (Rowe-Magnus and Manzel, 2002). Recruitment of exogenous genes is the most rapid

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adaptation against antimicrobial compounds and the integron functions provides gene cassette system that are perfectly suited to face challenges of multiple antibiotic treatment regimens.

1.6.7. Transposon mediated resistance

Prevalence of highly virulent *V.harveyi* strains harbouring a transferable chloramphenicol-resistance determinant together with other extracellular virulence factors may hamper the production of penaeid shrimp larvae (Abraham, 2006). The presence of the transposon Tn1721 carrying *tetA*, *tetR* genes and novel β -lactamases, antibiotic resistance determinants, makes them resist antibiotics. Antibiotic resistance can originate from gene mutations or by horizontal transfer between phylogenetically diverse bacteria. β -Lactamases, the enzymes that hydrolyze β -lactam antibiotics, are the main source of resistance to these drugs. Genes for β -lactamases may be found on chromosomes, plasmids, transposons, and integrons. TEM-1 β -lactamase gene is common among Gram-negative bacteria; it is one of the main causes of bacterial resistance to β -lactam antibiotics. The *blaTEM1* gene was detected in most of the isolates resistant to ampicillin and this gene is widespread in clinical as well as isolates from natural oligotrophic lake (Pontes *et al.*, 2009). Integrative and Conjugative elements (IECs) are diverse class of mobile elements found integrated to the chromosomes of Gram +ve and -ve bacteria. IECs encode conjugation systems that can transfer the excised DNA into a new host, where it integrates into the host chromosome by site specific recombination. Different IECs integrate into a variety of sites and encode diverse recombination, conjugation and regulation systems. They also carry genes encoding a variety of functions including catabolic pathways, antibiotic resistances, nitrogen fixation and phage mediated resistance mechanism (van der Meer and Sentchilo, 2003).

1.6.8. Resistance mediated by Plasmids

Bacteria that contain antibiotic resistance plasmids have shown to exhibit higher rates of survival in aquatic environments. Genes that encode resistance are the resistance determinants present in the R factor, whose products inactivate the antibiotics or prevent the antibacterial drug from contacting its target within the cell. A conjugative R factor plasmid in a *V.harveyi* strain virulent to *P.monodon* was reported by Harris (1993). This R factor conferred resistance to erythromycin, streptomycin, kanamycin, sulfafurazole and cotimoxazole. Bacteriocins, another class of plasmid-derived proteins produced by bacteria, exhibit antimicrobial activity against sensitive or closely related bacterial species. McCall and Sizemore (1979) reported a bacteriocin-like substance in *V.harveyi*, which caused lethality by a plasmid and was termed as harveyicin. Apart from their variable distribution, *Vibrio* plasmids show considerable microheterogeneity and modification of expression levels of some siderophore biosynthesis genes (Di Lorenzo *et al.*, 2003).

1.6.9. Bacteriophage mediated virulence

Phages thrive in bacterial population where they constantly transfer their genetic elements by horizontal gene transfer (Boyd *et al.*, 2001). Lysogenic cycle exhibited by phages confers virulence to *V.harveyi*. A temperate phage in *V.harveyi* VH1039 isolated from tea brown gill syndrome in *P.monodon* was identified as lysogenic siphovirus (Pasharawipas *et al.*, 1998). Oakey and Owens (2000) isolated a bacteriophage from a toxin-producing strain of *V.harveyi*, and termed it as VHML, which caused upregulation of certain bacterial extracellular proteins. VHML harbored by *V.harveyi* strains stimulate hemolysin production and excretion of proteins from cells and contributes to expression of virulence (Munro *et al.*, 2003; Austin *et al.*, 2003).

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1.6.10. Quorum sensing

Quorum sensing is a process that allows bacteria to communicate using secreted chemical signaling molecules called **Auto inducers** (Nealson and Hastings, 1979; Miller and Bassler, 2001; Natrah *et al.*, 2011, Ruwandeepika *et al.*, 2011). Quorum sensing is important for the regulation of population density dependent cellular processes in bacteria, including the production of antibiotics, virulent factors, conjugation, transformation, swarming behavior and biofilm formation (Fuqua *et al.*, 1994; Whitehead *et al.*, 2001). This mechanism enables a group to express specific genes only at particular population densities, but becomes unproductive when undertaken by individual bacterium (Xavier and Bassler, 2003). Three distinct autoinducers have been identified. **LuxR/I-type** systems are preliminarily used by Gram-negative bacteria, in which the signaling molecule is an acyl-homoserine lactone (AHL), the peptide signaling systems used primarily by Gram-positive bacteria is the **luxS/AI-2** signaling used for interspecies communication, and the **AI-3/epinephrine/norepinephrine** interkingdom signaling system. Quorum Sensing was first described in the regulation of bioluminescence in *V.fischeri* and *V.harveyi* (Nealson and Hastings, 1979; Henke and Bassler, 2004a, b, c). N-(β -Hydroxybutyryl) homoserine lactone is an autoinducer molecule of *V.harveyi*, which enables bacteria to monitor its own population and regulate virulence gene expression (Milton *et al.*, 1997, 2006). AI-2 is found to be produced by a large number of bacterial species, including *V.harveyi* which interacts with luminescence operon, composed of *luxCDABEGH* genes by the phosphorylation of regulatory protein *luxO* (Bassler *et al.*, 1997).

1.7. Treatment measures

1.7.1. Antibiotic usage and its drawbacks

Treatment with antibiotics and chemotherapeutics continues to be an unavoidable control measure in aquaculture industry, unless an alternative

replaces this traditional measure to control microbial agents in the culture systems. One of the most frequently used procedures to avoid the incorporation of undesirable bacteria is by antibiotic administration in the water or via live feed like *Artemia* (Brown, 1989; Touraki *et al.*, 1999). Antibiotics are also used in animal production system at sub-therapeutic level to boost food conservation. Teuber (1999) stated that the problem with drug resistance in human medicine will not be solved if there is a constant influx of resistant genes into human microflora via food chain. Feed with antimicrobial additives increases animal production and are beneficial on economic basis, but from a long term perspective their frequent use is questioned, as it is a matter of concern related to environment protection, animal welfare, and health.

Unconsumed feed, faeces etc., containing antibiotics reach sediment at the bottom of the rearing tanks, exerting selective pressure, altering composition of the sediment micro flora and promoting the overgrowth of antibiotic-resistant bacteria (Kim *et al.*, 2004 a, b). Disposal of antibiotics into the surrounding aquaculture sites has enhanced the number of antibiotic resistant bacteria, harbouring new and previously uncharacterized resistant determinants (Miranda *et al.*, 2002, 2003). The determinants of antibiotic resistance have the potential of being transmitted by horizontal gene transfer to bacteria of the terrestrial environment, including human and animal pathogens (Rhodes *et al.*, 2000). A strong association between the presence of integron and multiple antibiotic resistance (MAR) phenotype has been observed (Leversteinvan-Hall *et al.*, 2002). The development of multidrug resistant bacteria carrying the virulent-resistant genes is a serious threat to aquatic organisms, and is of concern with regards to the development of resistance to human pathogens. Residues of most commonly used antibiotics, such as erythromycin, oxytetracycline and chloramphenicol are found in shrimp meat which may cause health hazards in human on long term consumption (Bourne *et al.*, 2006). Virulent microbes re-enter the

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aquatic systems, establish biofilms on water pipes, air lines or in the animal gut, leading to clogging of the systems (Bourne *et al.*, 2006). When the resistant microbes establish themselves in the host body, there exist high chances of exchange of genetic information, especially the transfer of r-plasmids, enabling their resistance to further dosage of antibiotics (Bourne *et al.*, 2006).

The ever increasing concern over the potential harm to aquaculture systems is by the effluent discharge into receiving water bodies, bioaccumulation of harmful chemicals, contamination by aquatic products, which elevates human risks associated with storage and handling of these chemicals. Certain control measures and regulations to be followed by the producers are presented by FAO, 1995 in the “Code of conduct for Responsible Fisheries” to regulate the use of chemical inputs in aquaculture which has hazardous impact on human health and environment. Increase in the number of resistant varieties has resulted in the banning the use of certain antibiotics in aquaculture systems, necessitating the management strategies using immunostimulants, vaccines, probiotics, and phage therapy.

1.7.2. Probiotics as potential prophylactics

Chemicals including antimicrobial drugs, pesticides and disinfectants have been conventionally used to control diseases (Gomez-Gil *et al.*, 2000, Dahiya *et al.*, 2010). Abuse of these chemicals has brought forward development of environment-friendly aquaculture to resolve the problem and to develop sustainable aquaculture, and research on probiotics for aquatic animal health has been augmented (Gatesoupe, 1999, Castex *et al.*, 2008). Probiotics are viable bacteria that beneficially influence the host by improving its intestinal microbial balance (Wang and Xu, 2006, Vine *et al.*, 2006). The addition of antagonistic bacteria to water results in *vivo* disease reduction and /or reduction in the number of pathogenic bacteria in the culture systems (Moriarty, 1997, 1998, Gram *et al.*, 1999). Bacteria

occurring in aquatic ecosystems may have the ability to inhibit the growth of other microorganisms by producing antimicrobial substances. Addition of probiotics into culture ponds: 1) enhances decomposition of organic matter, 2) reduces nitrogen and phosphorus concentrations, 3) leads to greater availability of dissolved oxygen, 4) reduction of blue-green algae (Boyd *et al.*, 1984), 5) controls the level of ammonia, nitrite and hydrogen sulphide (Carmignani and Bennett, 1977), 6) lowers the incidence of disease and offers greater survival (Nogami and Maeda, 1992), 7) production of inhibitory compounds (Chythanya *et al.*, 2002), 8) competition for chemicals and available energy, 9) becomes a source of macro and micronutrients (Verschuere *et al.*, 2000a, b), 10) enhances competition to adhesion sites (Garcia *et al.*, 1997), 11) enhances immune response (Rengpipat *et al.*, 1998, 2000, 2003), 12) improve water quality and interaction with phytoplankton, and 13) increases enzymatic contribution to digestion and better shrimp and fish production (Tovar *et al.*, 2002).

The range of probiotics examined for use in aquaculture encompasses Gram positive and negative bacteria, bacteriophages, yeast and unicellular algae (Iriano and Austin, 2002a, b). Generally, probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. The identification of potential probionts has, however, expanded over the years to include species such as *A.hydrophila*, *A.media*, *B.circulans*, *B.subtilis*, *Carnobacterium*, *Clostridium butyricum*, Photosynthetic bacteria, *Saccharomyces boulardi*, *S.cerevisiae*, *Streptococcus*, *V.alginolyticus*, and *V.fluvialis* (Vijayan *et al.*, 2006, Zhou *et al.*, 2006, Kumar *et al.*, 2006). Various *Lactobacillus* spp., *Bacillus* spp. (Aly *et al.*, 2008b), *Carnobacterium* spp., *Aeromonas* spp. (Irianto and Austin, 2002b), *Micrococcus* spp. (Jayaprakash *et al.*, 2005), *Pseudomonas* spp. (Vijayan *et al.*, 2006; Holstrom *et al.*, 2003a), *Vibrio* spp. (Austin *et al.*, 1995, Balacazar *et al.*, 2007), yeast (Gatesoupe, 1999) and mixed cultures (Wang and Xu, 2006), etc in protecting fish and shellfishes from pathogens.

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Several studies on probiotics have been conducted during the last decades; however, the methodological and ethical limitations of animal studies make it difficult to understand the mechanisms of probiotic action, thereby revealing partial explanations. Nevertheless, some possible benefits linked to the administration of probiotics have already been suggested as: 1) competitive exclusion of pathogenic bacteria; 2) source of nutrients and enzymatic contribution to digestion; 3) direct uptake of dissolved organic material mediated by the bacteria; 4) enhancement of immune response against pathogenic microorganisms; 5) antiviral effects and 6) influence on water quality (Moriarty, 1998; Gomez-Gil, 2000; Balcazar *et al.*, 2006).

Screening of antagonism in environmental bacteria against pathogens by *in vitro* plate assay has been widely carried out (Verschuere *et al.*, 2000 a, b). However, selection based on properties such as adhesion, colonization to intestine, skin and other surfaces and growth parameters such as competition for nutrients, replication rate, production of antimicrobial substances, adaptation to the acidic environment of the gastrointestinal tract etc. has created importance in recent years (Vine *et al.*, 2004b). The hypothesis that preemptive colonization of the intestine and other portals of entry of pathogens by autochthonous bacteria with or without antagonism but with better adhesion, colonization and growth characteristics compared to pathogens can prevent pathogen invasions and improve survival (Hjelm *et al.*, 2004 a, b, Vine *et al.*, 2004a).

Currently, the four common methods employed to screen for inhibitory substances *in vitro* include; the double layer method, well diffusion method, cross-streak method and disc diffusion method. The principle behind all these methods is that a bacterium (the producer) produces an extracellular substance which is inhibitory to itself or another bacterial strain (the indicator). The inhibitory activity is displayed by growth inhibition of the indicator in the medium (Kesarodi-Watson *et al.*, 2008). Two major pitfalls of *in vitro* antagonism based selection of potential

probiotics are: 1) the other modes of probiotic activity such as immunostimulation, digestive enzyme production, competition for attachment sites or nutritional requirements, etc, need to be evaluated as the environmental conditions are widely different from that carried out on an agar plate in the laboratory, and 2) *in vitro* antagonism of a pathogen by a probiotic strain need not necessarily confer *in vivo* protection to the cultured animals. The property expressed *in vitro* may not be elicited under *in vivo* conditions. Gram *et al.*, (2001) found that *P.fluorescence* strain AH2 was inhibitory to *A.salmonicida* pathogenic to salmon *in vitro*. However, no protective effect was found when transferring the same probiotic to an *in vivo* challenge experiment. The methods to select probiotic bacteria for use in aquaculture include: 1) collection of background information (probiotics should not be pathogenic to the desired host, acceptable by host through ingestion, potential colonization and replication, reach the site of action within the host, preferably should not carry virulence resistant or antibiotic resistant genes), 2) acquisition of potential probiotics, 3) evaluation of the ability of potential probiotics to out-compete pathogenic strains, 4) assessment of the pathogenicity of the potential probiotics, 5) evaluation of the effect of the potential probiotics in host, and 6) economic cost/benefit analysis (Gomez-Gil., 2000). The putative probiotics can be added to the host or to its ambient environment through several ways: a) addition to the artificial diet, b) addition to the culture water, c) bathing, and d) addition via live feed (Austin *et al.*, 1995, Gomez-Gil,1998).

1.7.3. Immunostimulants

Short-term immunity is offered by vaccination or immunostimulation, due to the non-specific immune response of crustaceans. Immunostimulants are considered as an attractive alternative prophylactic measure to control microbial infections and stress reduction in shrimp (Logothetis and Austin, 1996). Immunostimulants are agents which stimulate the non-specific immune mechanisms on their own or specific

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immune mechanisms when coupled with an antigen. They activate the immune system of animals imparting resistance to infections caused by viruses, bacteria, fungi and parasites. Certain immunostimulants may act on animal cell membranes, making the surfaces more conducive to antigen uptake, while others can mimic animal's natural products, hence recognized as self by the host system. Wide range of substances such as microbial derivatives, plant or animal extracts, vitamins, hormones and synthetic chemicals have been reported to have immunostimulatory effects. Many synthetic polymers with repeated subunits, such as muramyl dipeptide, polynucleotides, polyadenylic polyuridylic acid, etc. have immunostimulatory effect on animals. Increase in growth and better survival in penaeid post-larvae were observed prior to the administration of *Vibrio* bacterins in the hatchery systems (Vici *et al.*, 2000). Complete Freund's adjuvant was the first immunostimulant used in animals to elevate the immune response. However, now FCA is used in conjugation with injection of bacterins. β -1,3-1,6- glucan (yeast cell wall extract) (Song and Sung, 1994) induces non-specific disease resistance to tiger shrimp especially against pathogenic vibrios, enhancing stress tolerance induced during hatching, transport and ammonia accumulation, suggesting the immunostimulatory effect of glucan (Song *et al.*, 1994). 1,3- β -D glucans incorporated into diet of brooder enhance the functional status of macrophages and neutrophils, modify immunosuppression and resistance to challenge with Gram -ve bacteria, enhance haemocyte- phagocyte activity, cell adhesion and superoxide anion production, and activate polyphenoloxidase in haemolymph (Scholz *et al.*, 1999). Vitamin-C is a popular immunostimulant added to diet of certain animals that have impaired antibody response as it enhances phagocytic engulfment of the pathogen and improves the immune mechanism. Immunostimulants and adjuvants can be administered before, with or after vaccines to amplify the specific immune response by elevating circulating antibody titers and number of plaque forming cells. In case where disease outbreaks are cyclical

and can be predicted, losses maybe reduced by activating the non-specific defense mechanisms and the immunostimulats maybe used in anticipation of events to prevent huge losses due to disease out break.

1.7.4. Vaccines

Adams *et al.*, (1991) have suggested the use of biological control methods such as vaccine and immunostimulants to prevent disease outbreaks and achieve sustainable production. During the last two decades, vaccination is carried out as a preventive method against various bacterial pathogens, leading to a lowered use of antibiotics dramatically (Somerset *et al.*, 2005). Though there is no specific memory in shrimps, a partial specificity in immune response was observed in the case of vaccine treated shrimps. However, vaccines composed of inactivated *Vibrio* species are reported to protect shrimps from vibriosis and to improve growth and survival of vaccinated shrimps. Li *et al.*, (2010) observed that the outer membrane protein (OmpK) can be used as an ideal vaccine against vibriosis caused to Orange-spotted grouper (*Epinephelus coioides*). Pereira *et al.* (2009) observed that cultivable penaeid shrimps can be protected against vibriosis, using formalin- killed *V.harveyi* vaccine. Maximum relative percentage survival at 1% vaccine concentration exposed for 5hrs, showed that vaccination is highly significant and enhances the resistance of shrimp post larvae to vibriosis. Genetically engineered subunit and DNA vaccines are being used increasingly in veterinary vaccine development. Vaccines absorbed to, held within or conjugated to particles or large molecules may aid uptake and efficacy of vaccines. Vaccines maybe coated to latex beads and bentonite or placed in liposomes or mixed with light oils and administered, leading to increase vaccine uptake, when the vaccines are given topically. Conjugation with haptens or small antigenic molecules to larger carrier molecules may also help immunogenicity of some vaccine, especially dealing with subunit, recombinant or synthetic vaccines that are expensive and difficult to prepare. Ergosan and Vibrimax vaccines showed

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significant enhancement in survival rate and promoted health status of *V.harveyi* and WSSV challenged juvenile stages of shrimp during the period of culture (Heidarieh, 2010). AquaVac™ Vibromax™ is a multivalent vaccine for shrimp that enhances resistance against a multiplicity of *Vibrio* species including *V.anguillarum* biotype I and II, *V.parahaemolyticus*, *V.harveyi* and *V.vulnificus*. AquaVac™ Ergosan™ is an alginate based immunomodulator extracted from marine algae. The active ingredients, including alginates and polysaccharides, are known to strengthen the full range of natural defense systems in fish. It is completely a natural product and as such is an accepted feed ingredient. A divalent vaccine containing formalinized cells and ECP of *V.alginolyticus* was developed by Morinigo *et al.*, (2002). A divalent vaccine prepared with formalinized whole cells and extracellular product of *Solea senegalensis* (Kaup), against *Vibrio harveyi* and *Photobacterium damsela* subsp. *Piscicida* has been attempted (Arijo *et al.*, 2005). Addition of sodium alginate in diet of white shrimp (*Litopenaeus vannamei*), cleared the pathogen *V.alginolyticus* and elevated immune parameters namely enhanced phagocytic index, phenoloxidase activity, respiratory burst and superoxide dismutase activity, but decreased glutathione peroxidase activity (Cheng *et al.*, 2005).

1.7.5. Phage therapy

Phages are abundant in marine ecosystems; comprising about 10^4 to 10^7 phage particle/ml. Temperate phages are also present in large numbers as lysogenic phages found in marine bacteria. Despite the extensive research carried out to control bacterial diseases in fish and shellfishes, still there exists a significant loss to farmers and potentially on wild stocks (Austin and Austin, 1999). One alternative control strategy that has received limited attention for aquaculture is the use of phage therapy; a concept first developed in 1918 by D'Herelle (Douglas, 1975). High specificity to target bacterial populations, effectiveness against multidrug resistant pathogens, spontaneous mutation of phages aiding rapid response to phage resistant

mutants, low production cost without any known side effects in comparison to antibiotics have boosted up the use of phage as therapeutics. Phage therapy has been explored with members of *Escherichia*, *Staphylococcus*, *Salmonella*, *Klebsiella*, *Proteus* and *Pseudomonas* for localized and systemic infections caused by *V.vulnificus*. *Siphoviridae* and *Myoviridae* phages are found specifically infect *V.harveyi*. Wu and Chao (1987) have described phage therapy against milkfish vibriosis. However, there are problems associated with phages as therapeutic agents, especially as phages are effective agents in the transfer of virulence factors or toxin genes (McGrath *et al.*, 2004). There are also other phage-associated toxins, of which the CTX cholera toxin (Davis *et al.*, 2000b), botulinum toxin (Brussow *et al.*, 2004), shiga-toxin (Strauch *et al.*, 2004) and diphtheria toxin (Brussow *et al.*, 2004) are well known. The extreme specificity of phages renders them ideal candidates for applications designed to increase food safety during the production process. Moreover, phages or phage derived proteins can also be used to detect the presence of unwanted pathogens in food or the production environments, which allows quick and specific identification of viable cells (Hagens *et al.*, 2007). Two important concerns need to be addressed: Are the effects of phages harmless upon consumption, and how can phage resistance is dealt with?

Phage typing is a popular tool to differentiate bacterial isolates, and is used in epidemiological studies with the aim of identifying and characterizing outbreak-associated strains. Although more sophisticated systems for differentiation are available, such as ribotyping, random amplified polymorphic DNA-PCR fingerprinting, or pulsed field gel electrophoresis of enzyme-digested DNA, the variable sensitivity to a set of bacteriophages (phage typing) remains a useful method because of its speed, relative simplicity, and cost-effectiveness. Various phage typing schemes exist for all common food-borne pathogens such as *Salmonella*,

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Campylobacter, *E. coli*, and *Listeria* (Majtanova and Majtan, 2006; Hopkins *et al.* 2004).

1.7.6. Quorum Sensing (QS) Inhibition

Since the appearance of antibiotic resistant bacteria has become universal, there is an increasing need for novel strategies to control infectious diseases like vibriosis. Biofilm forming bacteria have developed mechanisms to tolerate conventional antimicrobial treatments. The inactivation of the QS mechanism by the process called quorum quenching has resulted in the decrease of the pathogenicity caused by the luminescent vibrios. Cinnamaldehyde and its derivatives reduce virulence in vibrios by decreasing the DNA-binding activity of QS response regulator LuxR (Gilles *et al.*, 2008). QS inhibitors affect the starvation and reduce virulence in several *Vibrio* species interfering with LuxPQ (Gilles *et al.*, 2009). *Delisea pulchra*, a temperate marine macro red algae found in the Australian coast is capable of producing biologically active compounds (brominated furanone) with a broad range of antifouling and antimicrobial activity, especially inhibiting luminescence and toxin production in *V.harveyi*. This algae contains (5Z)-4-Bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone which inhibits swarming motility and biofilm formation in *Bacillus subtilis* and *E.coli* (Ren *et al.*, 2002). Extracts of *D.pulchra* have been found to reduce the growth rate of *S.aureus* and *S.epidermidis*, and inhibit the swarming of *P.mirabilis* (Gram *et al.*, 1996). *Bacillus thuringiensis*, *B.cereus* and *B.mycooides* were tested for AHL-inactivating enzymes. Exudates of pea seedlings inhibit QS in *Chromobacterium violaceum* but were found to activate QS in bacteria such as *Pseudomonas* and *Serratia*. The use of green water containing *Chlorella* during Tilapia culture (*Oreochromis*) has been suggested for minimizing *V.harveyi* (Fredson *et al.*, 2006). The ability of the green water grow-out culture of *P.monodon* to prevent outbreaks of luminous vibriosis was investigated by screening associated isolates of bacteria, fungi, phytoplankton, fish skin mucous for anti-*Vibrio* metabolites

(Gilda *et al.*, 2005). Natural furanone blocks QS regulated gene expression in *V.harveyi* by decreasing the DNA-binding activity of the QS transcriptional regulator LUXRvh and not by interacting with the receptor signal molecules. As furanones block all the 3 channels of *V.harveyi* QS transduction cascade, it is not necessary to develop different furanone compounds to protect the hosts. Furanones possess no or very small selective pressure on the bacteria, hence chances of development of resistance are lesser than conventional antibiotics, thus making these antipathogenic compounds an attractive sustainable biocontrol strategy (Defoirdth, *et al.*, 2007, 2008; Tinh, 2007).

1.8. Diagnostics for shellfish health management

Effective disease management of finfish and shellfish requires sensitive, accurate and rapid diagnosis without sacrificing the animals. The successful implementation of the diagnostic methods solely depends on the stage of disease progression at which the method is being used and the results are being interpreted. The effective control and treatment of diseases of aquatic animals require access to diagnostic tests that are rapid, reliable and highly sensitive. In many cases, post-mortem necropsy and histopathology have been the primary methods for the diagnosis of fish and shellfish diseases. Direct culture of pathogens is also widely used; however, these methods are time-consuming. Current diagnostic methods are categorized into 3 levels; **Level-1** includes farm or production site information and records on health management. **Level-2** uses specialised techniques such as microscopy, histopathology and antibody based diagnostic method. **Level-3** includes advanced techniques such as PCR based methods; multiplex testing using the Bio-Plex Protein Array System, ribotyping, and micro-array technology are bringing a new dimension to aquatic animal health control.

Histopathology provides information on host-pathogen interactions at structural and functional levels, detected using light microscope as signs

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of cloudy swelling, hydropic degeneration. Tissue necrosis, enteritis, fibrous encapsulation, nodule formation, xenomas etc are some of the common histopathological changes, based on which the pathogenic mechanisms of microbes, functional status of target organs, severity of a disease, cause of mortality and possible aetiology can be determined. However, these methods often lack specificity and many pathogens are difficult to detect when present in low numbers or when there are no clinical signs of disease. Histopathology being a non-specific diagnostic tool has certain limitations, but the advantages of using histopathology for aquatic animal health diagnostics and management outweigh its limitations.

Immunological techniques such as ELISA or dot-blot, agglutination (slide/latex); fluorescent antibody test (FAT/IFAT) (Adams 2004) are excellent diagnostic tools for pathogenic detection due to the specificity of antibody-antigen binding. Initially polyclonal antibodies (PABs) were used for detection, however, serious drawback of cross reactivity; availability in limited amounts and requirements of animals at various stage of antibody production have made this technique unpopular. Meanwhile, monoclonal antibodies (MAbs) overcome these limitations, hence are used as an effective immunological tool at different stages of disease detection. ELISA, one of the solid-phase enzyme immunoassay (EIA), is developed by application of the same antibody overlay principles used for the detection of antigens *in situ*. More sensitive ELISA detection system may be obtained by incorporating fluorescent substrates, alkaline phosphatase or beta galactosidase. Dot immunobinding assay first developed by Hawkes *et al.* (1982) using nitrocellulose is claimed equally or more sensitive than ELISA. Other diagnostic tools used include Immunoblotting, in which proteins are transferred from a gel after electrophoretic separation on to nitrocellulose membrane developed by Towbin *et al.* (1979) and Latex agglutination assay that detects antigen in a sample using antibody bound to a bead or other visible material. The main disadvantage associated with

Latex agglutination assay is that it is less sensitive than PCR and micro debris present along with the antigen are likely to affect the precipitation of latex particles leading to non-specific adsorption (Hu *et al.*, 2010). Also, unbalanced amounts of either antibody or antigen can give false-negative results. In addition, many bacteria have common or related antigens and some antisera may react with those bacteria, which have similar antigens. Another limiting factor is the size of the particles of the antigen, which must be opaque and large enough to cause turbidity and visible sediment. Immunofluorescence and Immunohistochemistry techniques employing antigen, labelled antibody or fluorescent dyes (fluorescein iso-thio-cyanate, rhodamine iso-thio-cyanate, etc) have gained important application. The main advantages of these tests are sensitivity and rapidity, but due to danger of cross-reactions, additional tests are required for confirmation. A significant problem with most fluorescence techniques is photobleaching. Loss of activity caused by photobleaching can be controlled by reducing the intensity or time-span of light exposure, by increasing the concentration of fluorophores, or by employing more robust fluorophores that are less prone to bleaching (e.g. Alexa Fluors or DyLight Fluors).

Advanced detection techniques such as PCR are largely qualitative and certainly more valuable diagnostic tools than mere qualitative detection methods, for revealing the severity of infection in culture ponds. Many molecular techniques are potentially faster or more sensitive than traditionally used methods such as culture, serology and histology. Molecular methods can circumvent problems inherent in the study of organisms for which no *in vitro* culture medium or methods are available, and have the potential to greatly increase the sensitivity of detection (Lightner, 2005). Many techniques are available to detect or exploit such genetic variations that denote subspecies or strains and can also assist in detecting the pathogens that are present in low numbers and can be used to differentiate antigenically similar pathogens. Various Competitive PCR methods have

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been developed for *in vivo* determination of even low levels of shrimp infectivity. DNA based techniques include various PCR targeted to specific conserved sequence of interest. RT-PCR, nested, real time, reverse cross blot PCR (rcb-PCR) and RT-PCR enzyme hybridisation assay (Cunningham, 2004) and multiplex PCR are being used to identify the pathogenic organisms at, above or below species level, allowing the diagnosis of infections in which the causative organisms are not easily cultured or are uncultivable. Real time PCR is more advantageous than traditional PCR as it involves both amplification and quantification of PCR product which is determined by FRET (Fluorescent Resonance Energy Transfer) using probes- Quencher and Reporter.

Ribotyping techniques are used in detection and identification of highly conserved bacterial ribosomal operons encoding for 16S and/or 23S rRNA genes by hybridizing with labeled probes (Thompson *et al.*, 2004). The technique has been developed based on the principle that all bacteria carry three operons which are highly conserved and are therefore useful for ribotyping. For the construction of oligonucleotide probes for hybridization, particular rRNA sequences that are species or group specific are used. An added advantage of ribotyping is its usefulness in differentiating bacterial strains into different serotypes. Hence, the probe DNA sequence used must be very specific for the virulent gene/factor associated with the pathogenicity.

DNA probe technology identifies a microorganism by probing its genetic composition, using variety of haptens such as biotin or digoxigenin and detection by antibody binding coupled to fluorescent, chemiluminescent or colorimetric detection methods. The use of probes in *in-situ* hybridization, applied to tissue sections or imprints, provides means to examine the location of pathogens within tissues and cells. Such methods have great advantages in applications in large-scale diagnosis of certain pathogens. Present advancement is the use of DNA and or antibody based Microarray

systems, which enable multiple pathogens to be screened and detected at one stretch on the array with the detectable signals. Plasmid profiling is another technique to type disease-causing aquatic vibrios (Le Chevalier *et al.*, 2003).

1.9. Existence of Beneficial forms

All aquatic organisms are exposed to a varied microflora inhabiting the aquatic ecosystem, having an easy access to host surfaces. Complex and highly evolved mechanisms aid in the interrelationship between aquatic organisms and their indigenous microflora including pathogens. Bacteria present in the intestine may either be beneficial to aquatic organism, in terms of nutritional value they impart (Campbell and Buswell, 1983) or in the prevention of colonization of gut by the host specific pathogenic bacteria (Westerdahl *et al.*, 1991).

1.9.1. Bacterial Communication

Certain beneficial forms of vibrios exist amidst the numerous pathogenic forms. Communication between bacteria and their hosts is an essential component of both beneficial symbiosis and pathogenic associations. Recognition of specific-cell surface receptor molecules and favorable adaptation to host internal environment favors bacterial colonization for normal growth, development, and function (Bassler *et al.*, 1993, 1994, 1997). Cell – cell communication by diffusible extracellular molecules or signals is evident in bioluminescent bacteria commonly found associated with marine animal tissues. These molecules enable antipredatory defense, defensive camouflage strategy and cryoprotection at lower temperatures to the host (Henke and Bassler, 2004a). Bacteria induce the host to secrete lipopolysaccharides (LPS), which trigger developmental response. Beneficial symbiotic *V.fischeri*, turns down the expression of the peroxidase gene in tissues but turns up the expression of this gene in tissues (specifically gills) when it acts as a pathogen (Winans and Bassler, 2002).

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1.9.2. Fermentative vibrios

Vibrionaceae exhibits two different fermentative patterns: mixed-acid fermentation and 2, 3-butanediol fermentation, which are distinguished by Voges-Proskauer (VP) and Methyl red (MR) tests. Microbes with mixed fermentative mode are MR -positive and VP- negative, while the other exhibits a reverse pattern. Mixed acid fermentors produce acetic, lactic and succinic acids along with ethanol, CO₂ and H₂ while butanediol fermentors produce less amount of acids, instead produce butanediol, ethanol, CO₂ and H₂ as the main products. Vibrios are ubiquitous in marine sediments, causing decomposition of organic matter via fermentative pathways, leading to the formation of small organic molecules, such as lactate, butyrate, propionate, acetate, formate, CO₂ and H₂, which serve as main substrates for sulfate reduction and partly for methane formation.

1.9.3. Chitin Degradation

Chitin, a (1→4)-β linked homopolymer of N-acetyl-D-glucosamine, is a widely found structural polysaccharide produced by various marine organisms, especially as an important element of crustacean exoskeleton. Chitin degradation is an important attribute of marine microbes, via complex pathways, including sensing, attaching, transporting and catabolism of natural chitin (Meibom *et al.*, 2004). When attached to zooplanktons and algal cells, vibrios can mediate degradation of highly polymeric substances, acting as important contributors to recycling of particulate matter. Partial hydrolysis of complex polymers occurs extracellularly prior to transport into periplasmic space. Chitinase activity is the most important enzymatic activity in marine environment, as annually about 10¹¹ tons of chitin is produced in marine systems in the form of zooplankton exoskeletons. The occurrence of vibrios in the gut of marine fauna, suggest the commensal role of vibrios causing the decomposition of organic matter. Great diversity of chitin structures present in the

environment necessitates bacteria to produce different forms of chitinase (Svitil *et al.*, 1997, 1998). Presence of Chitinase aids the invasion of pathogen and provides nutrients directly in the form of amino acids or by direct exposure to other host tissues for enzymatic degradation. Chitinase consists of a group of hydrolytic enzymes capable of breaking polymeric chitin to chitin oligosaccharides, diacetylchitobiose and N-acetylglucosamine, which are controlled by the expression of two genes (Thompson *et al.*, 2001). Specific attachment of *V.harveyi* to chitin is mediated by two peptides present in the outer membrane of the cells. Initial attachment is mediated by a 53kDa peptide and the other 150kDa peptide is induced by chitin for time-dependent attachment to the surface and cause pathogenicity to host animals (Montgomery and Kirchman, 1993, 1994). Analysis of gene sequence in vibrios shows that Chitinase genes only partially follows 16S rRNA gene phylogeny, suggesting that the deviation in phylogeny may be the result of lateral gene transfer.

1.9.4. Degradation of Polycyclic Aromatic Hydrocarbons (PAH)

Phenanthrene, a PAH present in coal tar and petroleum, formed as by product of petroleum refinery are degraded by many *Vibrio* species (Geiselbrecht *et al.*, 1996), suggesting that *Vibrio* species function as effective biodegraders in aquatic environments. Extracellular hydrolysis of complex polymers suggests an important cross-feeding mechanism in microbial communities (Riemann and Azam, 2002). Shifts in dominant and active forms of bacteria may strongly influence the pattern of polymer hydrolysis and cycling of dissolved organic matter in the aquatic systems. Geiselbercht *et al.* (1996) isolated polycyclic aromatic hydrocarbon degrading marine bacteria from Puget Sound sediments and phenotypically analysed them.

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1.9.5. Mucinase Production

Vibrios produce mucinase, a metalloprotease, which allows the bacteria to overcome the mucus barrier that covers the gastrointestinal epithelium. This mechanism is particularly exhibited by *V.cholerae* (Colwell, 2004).

1.9.6. Tetrodotoxin (TTX) production

Many *Vibrio* species, particularly *V.alginolyticus*, has been associated with TTX production and this toxin has been transmitted to puffer fish and other TTX containing organisms which initially were unable to produce the toxin but might have acquired the trait via the food chain (Lee *et al.*, 2000). *V.harveyi* is capable of producing marine toxins, such as tetrodotoxin and anyhydro-tetrotoxins (Simidu *et al.*, 1987). TTX binds to nerve cell sodium channels in myelinated and nonmyelinated nerves, hence has found widespread application as a research reagent in neurobiology, as pain killers, management of pain associated with withdrawal from heroin and other opioid drugs. Saxitoxin and TTX when mixed in small quantities have anaesthetic property (Simidu *et al.*, 1987).

1.9.7. Siderophore production

Siderophore-mediated iron transport system causes increase in virulence of some bacterial pathogens (Griffiths, 1987). Iron acquisition mechanism in pathogenic bacteria is limited to strong binding capacity of this element to high-affinity iron-binding proteins of animal body fluids. Many bacteria have complex system to transport iron into the cell in the form of siderophores, coupled with iron-repressible outer membrane receptors for siderophore/iron complex (Aznar *et al.*, 1989). Siderophores, low molecular weight Fe (III)-specific ligand function in receptor-dependent iron transport and act as virulence factors in animal and plant diseases. An increase in virulence of some pathogens is observed, when the host animals

were fed or injected with soluble iron. Siderophore are reported as major virulence determinant in *A.salmonicida*, *V.anguillarum*, *V.cholerae* and *A.hydrophila*. Siderophore activity in *V.harveyi* is linked to pathogenicity in vertebrates but not in invertebrates, mainly because of tight binding of iron by high-affinity iron-binding proteins such as transferrin and lactoferrin in serum and secretions (Crosa, 1989). Invertebrates seem to lack iron-binding compounds such as lactoferrin and transferrin (Owens *et al.*, 1996).

1.9.8. Bioactive compound production

Marine bacteria are known to produce brominated compounds and bromine has an effect on production of antibiotics (Marwick *et al.*, 1999). Several *Vibrio* species were isolated from marine sponge *Dysidea* sp. that synthesized cytotoxic and antibacterial tetrabromodiphenyl ethers (Marwick *et al.*, 1999). Other bioactive compound isolated from *Vibrio* include anticyanobacterial compound beta-cyanoalanine (Yoshikawa *et al.*, 2000), that could prevent algal blooms.

1.9.9. Enzyme production

Vibrio spp. produce a wide variety of extracellular proteases, including detergent-resistant alkaline serine exoprotease. Vibrios produce collagenase, important for various industrial and commercial applications including dispersion of cells in tissue culture. Vibrios are known to produce neutral protease called vimelysin. *Vibrio* proteases are responsible for breakdown of feather waste (Sangali and Brandelli, 2000).

1.9.10. Bioluminescence as a Reporter System

Bioluminescent bacteria are used to develop biosensors and as diagnostic devices for medicine, aquaculture and environmental monitoring. *lux* genes responsible for bioluminescence are cloned into a gene sequence or operon which turns functional when stimulated by a defined

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environmental feature. In case of toluene degradation, the enzymes are activated in the presence of toluene. When *lux* genes are inserted into a toluene operon, the engineered bacterium glows yellow-green in the presence of toluene and reports for its degradation (Applegate *et al.*, 1997). Also the lux system is responsible for monitoring and biodegradation of naphthalene (Burlage *et al.*, 1990), monitoring alginate production and many other compounds (Applegate *et al.*, 1997).

1.9.11. Role in Nutrient Cycling

Bacteria and protists play a major role in recycling of organic matter released from primary producers to supply regenerated nutrients, acting as sink for carbon lost during respiratory loss as CO₂. Through heterotrophic growth in organic substrates, vibrios contribute to nutrient cycling within the diverse habitat they occupy. Members of the family Vibrionaceae are involved in both uptake and mineralization of carbon, nitrogen and phosphorus and vibrios exhibit a population turnover and disproportionately contribute to ecosystem nutrient cycling. The extent to which vibrios cause nutrient recycling is a product of their abundance and activity.

Vibrios consume a wide array of carbon substrates and degrade them through extracellular digestion. Vibrios engage in both respiratory and fermentative metabolisms and transform organic carbon into cell materials and waste products. During aerobic and anaerobic respirations, 30 to 50% of organic matter is utilized for biomass formation. However, during fermentation, large amounts of metabolic end products are excreted. Organic acids, alcohols and H₂ formed as metabolic end products in some species, stimulate anaerobic food chains. Vibrios produce volatile organic compounds, such as acetone, during metabolism of leucine (Nemecek-Marshall *et al.*, 1999).

Nitrogen cycling involves series of microbial transformation stages, including: a) fixation of dinitrogen to organic nitrogen (N); b) dissimilatory

reduction of nitrate to produce nitrite or ammonia; c) nitrification of ammonia to nitrite or nitrate; and d) ammonification of organic nitrogen to ammonia (Herbert, 1999). However, vibrios participate in the transformation process except for nitrification. Nitrogen-fixing bacteria fix atmospheric nitrogen and have a profound effect on net community production by input of new nitrogen to nutrient-limited ecosystems. Nitrogen fixation is mediated by vibrios using the cytoplasmic nitrogenase enzyme complex (Coyer *et al.*, 1996). Occurrence of nitrate assimilation genes (*nasA*) in vibrios is correlated with ability to grow on nitrate as sole nitrogen source (Allen *et al.*, 2001). Many facultative aerobic bacteria can replace oxygen with nitrate as terminal electron acceptor via dissimilatory nitrate reduction. Several alternating electron acceptors, such as nitrate, fumarate and trimethylamine N-oxide, support anaerobic respiratory growth of vibrios (Proctor and Gunsalus, 2000). The dissimilatory reduction of nitrate to ammonia, carried out by marine vibrios is about 80% of overall nitrate consumption in marine sediments (Bonin, 1996). Remineralization of nitrogenous compounds such as nucleic acids, proteins and polyamino-sugars to simple carbon compounds and ammonia is a vital mechanism in nutrient recycling via microbial loop. Nutrient status and C:N ratio in the environment determine whether ammonia is incorporated into the microbial biomass or excreted into the environment. Mechanism of microbial consumption of polymeric nitrogenous compounds as both carbon and nitrogen sources involves extracellular hydrolysis of nitrogenous polymers to simpler subunits followed by the uptake of the monomers.

Vibrios have a number of extracellular enzymes that participate in degradation of phosphorus-containing macromolecules, also playing a role in the recycling of organic phosphorus into inorganic forms available for primary production. Inorganic phosphorus and polyphosphate ions, found in the dissolved marine phosphorus pools, can be directly utilized by microbes and phytoplanktons. The soluble non-reactive phosphorus pools, containing

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less macromolecular fractions such as monophosphate esters, nucleic acids and phosphonates are degraded extracellularly before utilization (Benitez-Nelson, 2000). Phosphate-generating exoenzymes are important for recycling organic phosphorus including alkaline phosphatase, phosphodiesterase and 5' nucleotidases (Hoppe, 2003). Alkaline phosphatase cleaves inorganic phosphate of phosphorylated compounds under neutral or alkaline conditions of the marine ecosystem (Roy *et al.*, 1982). 5' Nucleotidases degrade 5' nucleotides to inorganic phosphate and a base prior to its transport into the cytoplasm for subsequent metabolism. Hydrolysis of soluble nonreactive phosphorus by 5' Nucleotidases, supplies as much as half the phosphate required by planktons in coastal California waters (Benitez-Nelson, 2000). 3'5'cyclic nucleotide phosphodiesterases enable the metabolism of extracellular cyclic nucleotides such as cAMP. Such periplasmic enzymatic activity of vibrios in mineralizing organic compounds to inorganic compounds and carbon substrates for growth helps enrich local environments with dissolved pools of nutrients that can be utilized by several producer communities.

1.10. Vibriosis

In shrimp or prawn larval rearing systems and grow outs, vibriosis has been designated as systemic bacterial infection caused by several species of *Vibrio*, such as *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, etc. (Singh *et al.*, 1989, Lavilla Pitago *et al.*, 1990, Karunasagar *et al.*, 1994, Abraham and Manley, 1995). Among them *V. harveyi* has been designated as a potential pathogen in penaeids and *V. alginolyticus* in both penaeids and non-penaeids.

1.11. Significance of the present Study

In shrimp culture, vibrios are still the most important bacterial pathogen responsible for much of the losses. In penaeids, vibrios are known to be the pathogen causing systemic infections and necrotic appendages. The

present study focuses on vibrios especially *Vibrio harveyi* isolated from shrimp (*P. monodon*) larval production systems from both east and west coasts during times of mortality. A comprehensive approach has been made to work out their systematics through numerical taxonomy, confirm their identity through 16S rRNA gene sequence analysis and RAPD profiling to determine diversity and to segregate the virulent from non virulent isolates based on the presence of virulent genes as well as their phenotypic expression. The information gathered help develop a simple scheme of identification based on phenotypic characters, and to segregate the virulent from non-virulent strains of *V. harveyi*. The study also reveals the heterogeneity within *V. harveyi* clade.

Objectives

1. Phenotypic characterization and Numerical Taxonomy of vibrios
2. Genotypic characterization of vibrios based on RAPD profiling and analysis of housekeeping genes
3. Phenotypic expression of virulence- *in vitro* assays
4. Genotypic characterization using virulent and luminescent gene markers
5. Pathogenicity of *Vibrio harveyi* in animal model.

